Influence of meat source, pH and production time on zinc protoporphyrin IX formation as natural colouring agent in nitrite-free dry fermented sausages

Hannelore De Maere[1], Sylvie Chollet[2], Jos De Brabanter[3], Chris Michiels[4], Hubert Paelinck[1], Ilse Fraeye[1*]

[1]*Research Group for Technology and Quality of Animal Products, Department M²S, member of Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven Technology Campus Ghent, Gebroeders De Smetstraat 1, B-9000 Ghent, Belgium*

[2]*ICV – Institut Charles Viollette, EA 7394, ISA , Univ. Lille 1, INRA, Univ. Artois, Univ. Littoral Côte d'Opale, 48 Boulevard Vauban, Lille F-59000, France*

[3]*Department of Electrical Engineering (ESAT), member of the division STADIUS, Stadius Centre for Dynamical Systems, Signal Processing and Data Analytics, Kasteelpark Arenberg 10 – box 2446, B-3001 Leuven, Belgium*

[4]*Centre for Food and Microbial Technology, Department M²S, member of Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 23 box 2457, B-3001 Leuven, Belgium*

*corresponding author: Tel.: +32 9 331 66 17; fax: +32 9 265 87 24;
E-mail address: ilse.fraeye@kuleuven.be

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ABSTRACT

Nitrite is commonly used in meat products due to its plural technological advantages. However, it is controversial because of its detrimental side effects on health. Within the context of nitrite reduction, zinc protoporphyrin IX (Zn(II)PPIX) formation in meat products as natural red colouring agent has been suggested. This investigation presents the evaluation of naturally occurring pigments, namely Zn(II)PPIX, protoporphyrin IX (PPIX) and heme in nitrite-free dry fermented sausages in function of time, meat source (pork, horsemeat and a combination of both meat sources) and pH condition. In function of time, Zn(II)PPIX and PPIX were formed and heme content decreased. Higher pH conditions promoted Zn(II)PPIX and PPIX formation, whereas the influence of pH on heme was less clear. The use of horsemeat also promoted Zn(II)PPIX formation. Moreover, even similar amounts were formed when it was combined with pork. Product redness, however, could not be related to Zn(II)PPIX formation.

Keywords: nitrite-free meat products; natural colouring; meat source; pH condition; production time

1. Introduction

Meat colour is considered to be an important quality parameter of meat and meat products, influencing consumer's buying decision. Myoglobin (Mb) is predominantly responsible for the colour of meat, although low levels of hemoglobin and other heme proteins may also contribute to it. Specifically, the conjugated heme molecule (iron protoporphyrin IX) is responsable for the ability of Mb to absorb visible light. This heme is located in a hydrophobic cleft of the protein where only small ligands, such as oxygen (O_2), nitric oxide (NO), carbon oxide,... have ready access (Devine & Dikeman, 2004). Colour manifests itself in many different shades depending on the nature of ligand attached to iron and the oxidation state of iron. O_2 can only bind to iron in the ferrous redox state (Fe(II)) forming the cherry-red oxymyoglobin (OMb), in absence of O_2 no ligand is bound to Fe(II) whereby the purplish deoxymyoglobin (DMb) is formed, whereas water is bound to iron in the ferric redox state (Fe(III)) with formation of the brownish metmyoglobin (MMb) (Lindahl, 2005). The occurence of these Mb forms depends on *e.g.* temperature and O_2 pressure. But also other parameters, such as Mb concentration, moisture and fat content have an effect on colour (Adamsen, Møller, Laursen, Olsen, & Skibsted, 2006).

Sodium nitrite is commonly used in meat products. Besides its antimicrobial (especially against *Clostridium botulinum* strains) and antioxidant properties, its contribution to an acceptable flavour and taste, nitrite is mainly used as colouring agent. After reduction of nitrite, Mb will form a complex with NO, resulting in the red pigment nitrosylmyoglobin (NOMb) (Honikel, 2008). Despite the many technological advantages, the addition of nitrite (E249, E250) is legally restricted to 150 mg/ kg (expressed as $NaNO_2$/ kg) in most meat products because of their detrimental side effects on health (Regulation (EC) No 1333/2008; lastly amended in 2015; Schuddeboom, 1993; Skibsted, 2011). In addition, the consumer

shows a strong desire to avoid all artificial food additives (so-called E-numbers) in the daily diet. In parallel, it is unconceivable to present a grey slice of meat product to the consumers. As such, nitrite reduction is already some years a matter of interest, whereby colour formation in meat products without the use of nitrite or other artificial colouring agents is one of the challenges[1].

In this context, zinc protoporphyrin IX (Zn(II)PPIX), a natural red pigment found in nitrite-free dry cured hams, has been investigated (Adamsen et al., 2006; Takenati, Mutsumi, Mizutani, Uebayashi, Numata, & Ohgari, 2007; Wakamatsu, Nishimura, & Hattori, 2004a). The exact formation pathway of Zn(II)PPIX is still disputed, although it is nowadays generally assumed that Zn(II)PPIX originates from Mb whereby the iron in the heme moiety is replaced by zinc (Chau, Ishigaki, Kataoka, & Taketani, 2011; Takenati et al., 2007). Enzymatic formation with ferrochelatase (FECH) includes both the removal of Fe(II) from heme and the insertion of zinc into protoporphyrin IX (PPIX) (Chau, Ishigaki, Kataoka, & Taketani, 2010). FECH activities have been detected in mammals, bacteria and yeast (Dailey, Dailey, Wu, Medlock, Wang, Rose, & Wang, 2000; Medlock, Swartz, Dailey, Dailey, & Lanzilotta, 2007). Wakamatsu, Okui, Ikeda, Nishimura, & Hattori (2004b) and Wakamatsu, Uemura, Odagiri, Okui, Hayashi, Hioki, Nishimura, Hattori (2009b), however, suggested a minor role of bacteria for the formation of Zn(II)PPIX in pork and dry cured ham. Formation of Zn(II)PPIX by endogenous FECH was first demonstrated by Wakamatsu et al. (2004b). Additionally, also a non-enzymatic mechanism cannot be fully excluded, suggesting a parallel non-enzymatic and enzymatic formation of Zn(II)PPIX in meat products (Becker, Westermann, Hansson, & Skibsted, 2012).

Colour of nitrite-free dry cured hams is attributed mainly to the presence of Zn(II)PPIX (Wakamatsu et al., 2004a; Wakamatsu, Odagiri, Nishimura, & Hattori, 2009a). A steady

[1] We are well aware that omission of nitrite in dry fermented sausages results in reduced microbial safety, especially with regard to *Clostridium botulinum* which can cause food poisoning. However, further investigation on food safety was outside the scope of this work.

increase in redness intensity of Parma ham during processing could be seen and due to colour differences between muscles with equal moisture losses, it was assumed that the increase in redness could not only be a consequence of dehydratation. Until now, however, no clear relation between colour and Zn(II)PPIX formation in meat products could be demonstrated (Adamsen et al., 2006; Parolari, Benedini, & Toscani, 2009). Degradation of heme may complicate colour formation in the nitrite-free meat products (De Maere, Fraeye, De Mey, Dewulf, Michiels, Paelinck, & Chollet, 2016a; Wakamatsu et al., 2009b).

In order to evaluate the formation of Zn(II)PPIX mainly pork has been used (Adamsen et al. 2006; Chau et al. 2010; Ishikawa, Kawabuchi, Kawakami, Sato, Numata, & Matsumoto, 2007; Wakamatsu et al. 2004a). In contrast, De Maere, Chollet, Claeys, Michiels, Govaert, De Mey, Paelinck, & Fraeye (2016b) compared different meat sources in their ability to form Zn(II)PPIX *in vitro* and related this pigment formation to several intrinsic parameters. This investigation revealed that endogenous enzymatic Zn(II)PPIX formation is species-dependent, whereby horsemeat, better than pork, showed very good ability to form Zn(II)PPIX and that zinc chelatase activity, followed by heme and zinc content, was the most important factor to explain the variation in Zn(II)PPIX formation between the investigated meat sources.

Furthermore, hardly any research focussed on Zn(II)PPIX formation in nitrite-free meat products other than dry cured hams. In this respect, De Maere et al. (2016a) recently studied Zn(II)PPIX formation in nitrite-free porcine dry fermented sausages. They found that Zn(II)PPIX formation and product redness were significantly correlated. Zn(II)PPIX, however, was only able to form at pH values higher than 4.9 and after an extensive drying period up to 177 days, indicating that both pH and production time are crucial factors for its formation.

Based on both recent studies, it was hypothized that the use of horsemeat in the production of dry fermented sausages may be a promising route to accelerate Zn(II)PPIX formation and

improve product redness. Therefore, the goal of this study was to examine the effect of meat source on Zn(II)PPIX formation and relate the Zn(II)PPIX formation to colour development in dry fermented sausages made of pork and horsemeat. By using horsemeat, however, a more drastic pH decline could be expected upon fermentation, due to the presence of higher concentrations of residual sugar. The concentration of glycogen and reducing sugars, including glucose, in horsemeat *post mortem* have been reported as > 5 mg/ g and > 0.5 mg/ g, respectively (Gill, 2005). In pork, *post mortem* glycogen contents < 1.78 mg/ g were reported (Choe, Choi, Lee, Shin, Ryu, Hong, & Kim, 2008). The expected strong pH decrease upon fermentation could be disadvantageous for Zn(II)PPIX formation. Therefore, also dry fermented sausages containing both horsemeat (having the advantage of high zinc chelatase activity, as shown by De Maere et al., 2016b) and pork (having the advantage of a lower amount of sugars, hence a higher pH upon fermentation) were included in the study.

In summary, this study presents the production of nitrite-free dry fermented sausages based on pork, horsemeat and a 50/50 combination of pork and horsemeat at two different pH conditions, whereby pigment and colour formation were evaluated in function of meat source, pH and production time by linear mixed modelling.

2. Material and methods

2.1. Dry fermented sausages

Nitrite-free dry fermented sausages were prepared as described in De Maere et al. (2016a). In total, six treatments were made *in duplo*. The shoulder meat fractions originated from single homogeneous batches of pork, horsemeat or a 50/50 combination of both meat sources. For each of the three meat source treatments, 0.00% and 0.70% dextrose was added to the meat batter in order to obtain two significantly different pH conditions during processing, a high and a low pH condition, respectively. An overview of the different nitrite-free dry fermented sausage preparations and the corresponding codes is given in Table 1.

2.2. Sampling

Core samples of sausages of each treatment were taken at different points in time during the production process, more specifically at production day (day 0), after the fermentation process (day 3), after the initial drying period which is normally the end of production for semi-dry Northern type dry fermented sausages (day 21), and during an extended drying period (day 42, 63, 84, 105, 126 and 168). General analyses for process monitoring, by means of weight losses, pH, dry matter (DM) and water activity (a_w), were performed immediately at each sampling day. Microbial analyses were performed at sampling days 0, 3 and 21. Also immediately after sampling, colour was measured and Zn(II)PPIX and/ or PPIX formation was screened. Other samples were frozen at -24 °C until quantitative analysis was performed of PPIX, Zn(II)PPIX and total heme. The latter analyses were only performed at sampling days 0, 21, 63 and 168 (cf. *infra*). All measurements were done *in triplicate*, only colour was measured six times.

2.3. Analysis

2.3.1. General analyses

Weight losses, pH and a_w were analysed as described in De Maere et al. (2016a).

2.3.2. Microbial count

Samples were aseptically homogenized with a stomacher (Masticor Classic 400, IUL Instruments, Barcelona, Spain). Decimal dilution series were prepared with sterile ringer solution (Oxoid, Basingstoke, England) and plated with a spiral plater (Eddy Jet, IUL Instruments). Total aerobic count (TAC) was analysed on plate count agar (PCA, Merck, Darmstadt, Germany) incubated at 26 °C for 48 hours, lactic acid bacteria (LAB) were analysed on de Man, Rogosa and Sharpe agar (MRS, Merck) incubated with a double layer at 30 °C for 72 hours, Staphylococci on mannitol salt agar (MSA, Merck) incubated at 30 °C for 48 hours and *Enterobacteriaceae* on violet red bile glucose agar (VRBG, Biokar, Beauvais, France) incubated with a double layer at 30 °C for 48 hours. Data are expressed as log colony forming units (cfu) per gram meat sample.

2.3.3. Determination of PPIX, Zn(II)PPIX and total heme pigments

A screening method was used for the fast detection of the fluorescent Zn(II)PPIX and/ or PPIX on transverse slices of meat products. Generally, the fluorescence emission obtained after irradiation of meat slices with purple LED light of 420 nm in a darkened room was visualized via image analysis. A darker picture is assumed to represent a higher amount of Zn(II)PPIX and/ or PPIX. PPIX and Zn(II)PPIX were quantified simultaneously by means of High Pressure Liquid Chromatography (HPLC) with fluorescence detection. Total heme content was determined spectrophotometrically. These methods have been described extensively in earlier published work by De Maere et al. (2016a).

187 2.3.4. Colour measurements

188 A portable Miniscan EZ 4500L 45°/0° (Hunterlab, Murnau, Germany) with 8 mm viewing
189 area size, illuminant D65 and 10° standard observer was used to register the *L**, *a** and *b**
190 values (based on CIE, 1976).

191 *2.4. Data analysis*

192 Differences in pH, weight losses and a_w between the six different treatments at each sampling
193 day were assessed using a two-way ANOVA (Christensen, 2015) at a significance level of P
194 < 0.05. A Tukey correction was used to account for multiple testing (Hochberg & Tamhane,
195 1987) (IBM SPSS Statistics 21.0, Chicago, USA).

196 As already described in detail (De Maere et al., 2016a), Zn(II)PPIX, PPIX and total heme on
197 the one hand, and *L**, *a** and *b** on the other hand, were analyzed using a linear mixed model
198 (Verbeke & Molenberghs, 2013) that included factors for meat source, pH condition, time and
199 their two-way and three-way interactions (SAS version 9.4 with SAS/STAT 14.1).

200

3. Results and discussion

3.1. pH

Means ± SE of pH are shown in Table 2. At day 0, no significant differences in pH of all prepared meat batters were measured. This was expected as, despite the differences in glycogen levels (Gill, 2005; Choe et al., 2008), similar ultimate pH values have already been reported in literature (Devine & Dikeman, 2004; Gill, 2005; Litwinczuk, Florek, Skalecki, & Litwinczuk, 2008). After fermentation, however, decreases of 0.40 and 1.10 pH units were observed in the pork-high and pork-low treatments, respectively. Due to the presence of more residual sugars (Gill, 2005), stronger decreases were obtained in the horse-high and horse-low treatments, namely 0.95 and 1.26 pH units, respectively. For the combi-high and combi-low treatments, intermediate decreases of 0.78 and 1.15 pH units were seen, respectively. For each meat source, the differences in pH after fermentation between sausages based on addition of different dextrose concentrations were statistically significant. It is important to stress, however, that within the experimental setup, the actual pH values between the meat sources vary, even within the treatment "high pH" or "low pH". This must be kept in mind throughout the interpretation of results obtained. During the further processing, more specifically at production days 21, 42, 63, 84, 105, 126 and 168, the differences in pH between the two pH conditions, but also between the different meat sources used, remained, despite the overall re-increase of pH in function of production time as result of proteolysis (Toldra, 2008).

3.2. Weight loss and a_w

Mean values ± SE of a_w and weight losses are shown in Table 2. The use of different meat sources for the preparation of nitrite-free dry fermented sausages and the obtained pH conditions did not affect the weight losses up to day 105. From that day, however, higher weight losses occurred in the horse-low treatment compared to the combi-low and pork-low treatments, respectively. The use of different meat sources had also no clear influence on the

a_w-decline during drying. For the sausages with high pH conditions, however, a_w was generally higher than those with low pH conditions except in those only made with pork. Differences in a_w might be explained by the coagulation of meat proteins at lower pH conditions (Toldra, 2008). The reason why pH did not affect the pork treatments, could not be explained. As a function of time, a_w decreased gradually due to the persistent drying conditions. Weight losses of the sausages increased, although more pronounced in the beginning of the drying process. During the first 3 days, only a slight decrease of weight of the sausages occurred, which can be attributed to the high relative humidity (95 % RH) during fermentation.

3.3. Screening of Zn(II)PPIX and/ or PPIX formation as selection tool for the further quantification of natural pigments

Figure 1 shows the red fluorescence emission of the six treatments at multiple time points during the production process.

At day 0, only little and similar red fluorescence was observed for all treatments. After fermentation, an overall slight increase in red fluorescence emission was seen, independent of the meat source used and pH condition. The increased temperature of 24 °C during fermentation, favouring FECH activity, probably plays a role here (Chau et al., 2011; Wakamatsu, Okui, Hayashi, Nishimura, & Hattori, 2007). At day 21, clear fluorescence appeared in the pork-high treatment, followed by the combi-high and the horse-high treatments. Compared with day 3, no differences in fluorescence emission were seen for all treatments with low pH conditions. Hence, higher pH values correspond with higher red fluorescence emissions. At day 63, even higher red fluorescence emission could be seen in the treatments with high pH conditions. However, a shift was seen, whereby darker pictures were obtained in the combi-high treatment compared with the pork-high treatment, despite the higher pH values of the latter. During the further production process, however, the differences

between the pork-high and horse-high treatments became less pronounced in function of time (mainly because of a decreasing fluorscence emission in the cores of the pork-high treatment). These observations could already indicate that our hypothesis, namely that producing nitrite-free dry fermented sausages based on both pork and horsemeat could improve Zn(II)PPIX formation due to the achievement of optimal pH values and zinc chelatase activities, was promising.

From day 63, no differences in red fluorescence emission were seen for all sausages at low pH condition in the core. However, clear changes with increasing red fluorescence emissions in function of time were noted in the outer regions (periphery). This increase was most pronounced in the pork-low treatment. In these samples, pH was measured in the periphery (results not shown), showing higher pH values than those measured in the core. The observation of more red fluorescence emission in the periphery compared to the core corresponds to higher pH values in the periphery, despite the expected more aerobic circumstances which was considered to inhibit Zn(II)PPIX formation in meat products (Wakamatsu et al., 2004b; Wakamatsu et al., 2006).

The screening method offers the opportunity to easily and qualitatively assess the formation of Zn(II)PPIX and/ or PPIX. Based on the obtained observations, it was chosen only to quantify Zn(II)PPIX, PPIX and total heme pigments at sampling day 0, 21, 63 and 168, as these time points were assumed to deliver the most crucial information.

3.4. Evolution of Zn(II)PPIX, PPIX and total heme content in nitrite-free dry fermented sausages based on different meat sources and at different pH conditions

The concentrations of Zn(II)PPIX, PPIX and total heme as a function of meat source, pH condition and production time in nitrite-free dry fermented sausages, are presented in Table 3.

Zn(II)PPIX analysis

At day 0, no differences in initial Zn(II)PPIX content between all treatments was seen. Zn(II)PPIX formation occurred in the first 21 days of processing, in exception of the pork-low treatment. During the further production process, Zn(II)PPIX formation was observed in the horse-high, horse-low and combi-high treatments. However, no increase in Zn(II)PPIX could be seen in the pork-high treatment. This is in contrast to the results obtained in the earlier published work (De Maere et al., 2016a), whereby a remarkable formation of Zn(II)PPIX was seen after an extensive drying period of 177 days. It was stated that the factor time, potentially related to partial Mb denaturation, is of major importance for the formation of Zn(II)PPIX (Grossi, do Nascimento, Cardoso, & Skibsted, 2014; Paganelli, Grossi, Dores-Silva, Borges, Cardoso, & Skibsted, 2016). The reason why in this study the highly increased Zn(II)PPIX formation at a later stage in the production process did not occur in the nitrite-free porcine dry fermented sausages at a similar high pH level is not clear and requires further investigation. Possibly, variation in raw material (meat batch) or differences in starter culture development, resulting in differences in enzymatic activity (zinc chelatase or proteolytic activity causing Mb degradation) can be at the basis of this observation. Within the high pH treatments, Zn(II)PPIX formation was the poorest in the pork-high treatment, but showed to be equal in the horse-high and combi-high treatments. These results revealed that formation of Zn(II)PPIX in nitrite-free dry fermented sausages at the high pH condition can be significantly ameliorated if horsemeat is used. Moreover, a similar effect is obtained if only 50% of the meat was based on horsemeat.

In all cases, the pH condition of the sausages influenced the formation of Zn(II)PPIX, with significantly higher amounts at the highest pH conditions. This can be explained by the pH dependence of FECH activity, with pH optima around 5.5 for porcine FECH in meat-based models (Ishikawa, Yoshihara, Baba, Kawabuchi, Sato, Numata, & Matsumoto, 2006;

299 Wakamatsu et al., 2007). Specific pH optima for equine FECH, however, could not be found
300 in literature.
301 Not included into the statistical experiment, but nevertheless worth mentioning, is that no
302 significant differences in pH (at the majority of sampling days) were obtained between the
303 pork-low and the horse-high treaments, which enables us to compare Zn(II)PPIX formation in
304 sausages based on different meat sources at similar pH values. Remarkable differences in
305 Zn(II)PPIX formation were observed between the pork-low and horse-high treatments. The
306 horse-high treatment revealed more Zn(II)PPIX formation than the pork-low treament.
307 Influence of meat source on Zn(II)PPIX formation has already been shown in De Maere et al.,
308 (2016b), whereby horsemeat showed better ability to form Zn(II)PPIX *in vitro* compared to
309 pork, which was explained mainly by its higher zinc chelatase activity, but also by its higher
310 heme and zinc content.
311 For all this, however, it is assumed that the influence of bacterial population on Zn(II)PPIX
312 formation is minimal or similar. For both treatments, 9 log cfu/ g on PCA agar plates, 9 log
313 cfu/ g on MRS agar plates and 6 log cfu/g on MSA agar plates were counted at day 21,
314 indicating that the starter culture was present in equal amounts. *Enterobacteriaceae* did not
315 exceed the quantification limit of 3.5 log cfu/ g. Of course, more investigation about the
316 influence of bacteria on the formation of Zn(II)PPIX should be done and was not included in
317 this study.
318 *PPIX analysis*
319 No diffences in initial PPIX concentration were found. For the pork-high treatment,
320 increasing PPIX concentrations were found up to day 63, but at day 168 a decreased
321 concentration could be seen. PPIX formation occured during the first 21 days of processing
322 and stabilized during the further drying period for the horse-high treatment. For the combi-
323 high treatment, PPIX formation occured during the first 21 days of processing and stabilized

temporarily until a decrease was seen at day 168. No differences in PPIX formation between the different meat treatments were seen at day 21 and 63. But due to the decrease of PPIX in the pork-high and combi-high treatments at day 168, the concentration of PPIX was found to be higher in the horse-high treatment. At low pH conditions, PPIX formation did not occur in any meat treatment. Similar to Zn(II)PPIX formation, the pH condition of the sausages influenced the formation of PPIX, with significantly higher amounts at the highest pH conditions in all cases in exception of the combi treatment at day 168. An accumulation of PPIX at high pH conditions has already been noticed in porcine nitrite-free dry fermented sausages (De Maere et al., 2016a). In the current study, also an increased PPIX formation was seen, although not as pronounced as in previous study. In accordance to the results obtained for Zn(II)PPIX, a higher PPIX concentration was found in the horse-high treatment than in the pork-low treatment, having a similar pH evolution during processing.

Total heme analysis

Depending on the meat source used, the meat batter showed clear differences in total heme amount, with the pork treatments having the lowest total heme concentrations, the horse treatments having the highest total heme concentrations and the combi treatments resulted in intermediate total heme concentrations. The higher concentrations of total heme in horsemeat compared to pork corresponds to what was already described in literature (De Maere et al., 2016b). During the production process, generally a decrease in total heme was seen (except for the combi-low treatment), although the course of this decrease differed between treatments, with no clear effect of meat source or pH treatment. A reduction of total heme concentrations has been already observed in dry cured ham and dry fermented sausages (Chasco, Lizaso, & Beriain, 1996; De Maere et al., 2016a; Wakamatsu et al., 2009b). The only exception was seen in the horse treatments, whereby a strong decrease in total heme was

seen between day 21 and 63, but a re-increase at day 168. However, these findings could not be explained.

Although a substitution reaction is assumed for the formation of Zn(II)PPIX, no clear conclusion could be drawn about the decreasing heme pigments and the formation of Zn(II)PPIX and PPIX. In this study, the sum of the three pigments generally decreased in function of time, implying that more total heme breakdown occured than needed for the substitution process alone.

3.5. Colour formation in nitrite-free dry fermented sausages based on different meat sources and at different pH conditions

Mean values of L*, a* and b* as a function of meat source, pH condition and production time are shown in Table 4. In addition, analysis for a* was adjusted for Zn(II)PPIX, PPIX, total heme and for all three simultaneously in order to relate the investigated pigments to the colour measurements.

L analysis*

Between the meat sources used, highly different results could be seen, with the pork treatments having the highest *L** values (being more bright), the horse treatments having the lowest *L** values (being more dark) and the combi treatments having intermediate *L** values. These differences remained during the further production process. During the first 21 days of processing, a slight increase in *L** value was observed in all treatments, which was significant in case of the horse-low treatment. During further production, *L** tended to decrease, which can probably be attributed to a decrease in moisture content, resulting in a darker product. Also no clear effect of pH on *L** could be found, as the significant differences showed no clear trend in this regard.

a analysis*

At day 0, the *a** values of the meat batter based on horsemeat were found, although not significant in case of the high pH treatment, higher than those based on pork. In contrast to *L**, the results of *a** were highly influenced by the fermentation process whereby the sausages evolved to a less red (decrease of *a**) colour, which can be attributed to the formation of higher concentrations of MMb (Adamsen et al., 2006). The more drastic decrease in *a** of the sausages based on horsemeat during fermentation could imply that higher amounts of MMb were formed, which could be a result of its higher Mb content (Feiner, 2006). For the pork and combi treatments, an increased *a** value was seen at the later phase of the production process, namely at sampling day 168. This was not the case for the horse treatments, which in contrast even decreased during further processing. Differences in *a** between the two pH conditions were mainly seen in the pork and combi treatments, with a higher *a** value at the highest pH condition. This was not seen for the horse treatments. The latter exhibited very low *a** values during production, in comparison with the sausages whereby pork was used as a meat source.

The linear mixed model also allowed to relate the investigated pigments with *a**. None of the pigments, however, was found to have an effect on *a** values. More specifically, no effect of Zn(II)PPIX on *a** (P = 0.9736) was found during production of nitrite-free dry fermented sausages based on different meat sources and at different pH conditions. Zn(II)PPIX formation occured mainly in the horse and combi treatments. Redness, however, was very poor in the horse treatments, was higher in the combi treatments and was the highest in the pork treatments. Also in earlier studies, it was not easy to relate the presence of Zn(II)PPIX to instrumental colour measurements in meat products (Adamsen et al., 2006; Parolari et al., 2009). In our previous study, *a** of the porcine sausages was significantly related to the content of Zn(II)PPIX (De Maere et al., 2016a), which suggested that Zn(II)PPIX influenced redness of the meat products. In the latter study, at the end of production a huge increase of

Zn(II)PPIX formation was seen at the highest pH condition, with Zn(II)PPIX concentrations up to 125.69±5.66 nmol/g DM at day 177. In those sausages, an *a** value of 13.03±0.19 was measured. In contrast, as discussed in section 3.4, this huge increase of Zn(II)PPIX formation at the highest pH condition was not observed in the current study, the Zn(II)PPIX concentration in porcine sausages at high pH was only 11.63±0.50 nmol/g DM at day 168. Still, an *a** value of 12.74±0.46 was obtained, which is almost as high as the values obtained in previous study. In contrast, the horse-high and combi-high treatments reached at day 168 Zn(II)PPIX concentrations of 59.51±2.47 nmol/g DM and 61.20±2.25 nmol/g DM, respectively, but these concentrations did not result in increased *a** values. On the contrary, *a** values were very low, especially in the case of sausages prepared with horsemeat. Therefore it can be concluded that no relation could be found between the pigments quantified in this study and redness in these dry fermented sausages, and that the Zn(II)PPIX formed did not act as a natural coloring agent. Potentially, formation of MMb during fermentation, especially in sausages prepared with horsemeat, may overrule the coloring effect of Zn(II)PPIX.

*b** analysis

Differences in *b** values were found between the meat batters at day 0, with meat batters based on pork showing the highest *b** values, meat batters based on horsemeat showing the lowest *b** value and meat batters based on the 50/50 combination of both meat sources showing intermediate values. However, these differences were rather small. Similar to the *a** values, *b** values were also influenced by the fermentation process, whereby the sausages evolved to a less yellow (decrease of *b**) colour. This can probably be attributed to the formation of higher concentrations of MMb (Adamsen et al., 2006). Similarly as in case of the a* value, this decrease in b* was again stronger for sausages prepared with horsemeat, which may be related to its higher Mb content (Feiner, 2006), resulting in stronger formation of

MMb during fermentation. In function of time, *b** values of the pork treatments increased, but increased and remained stable or decreased again for the horse and combi treatments. As a result, *b** values remained highest in the former treatments during the further production process. However, the latter changes in b* values during processing were limited. Significant pH effects were found, but no clear trend could be seen. It can be concluded that differences in b* value were limited and could mainly be attributed to probable differences in MMb formation during fermentation.

4. Conclusion

Zn(II)PPIX formation is significantly promoted by a higher pH and by the use of horsemeat. Both effects can probably be attributed to higher zinc chelatase activity. However, Zn(II)PPIX contents obtained after long processing times were lower compared to a previous study based on pork. Due raw material variation and/or complex processes during fermentation and ripening, a stable, standardized formation of Zn(II)PPIX in nitrite-free dry fermented sausages cannot yet be guaranteed.

None of these pigments measured (Zn(II)PPIX, PPIX and heme) had an effect on the redness (*a** values) of the sausages. The higher concentrations of Zn(II)PPIX obtained in nitrite-free dry fermented sausages based on horsemeat, but also by combining pork and horsemeat, did not act as a natural colouring agent. This indicates that the redness of these sausages is determined by other factors that were not quantified in this study, such as MMb content. Therefore, a better insight in the factors determining colour of nitrite-free meat products remains indispensable for meat industry for investigating the elimination of nitrite as colouring agent in meat products.

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Table 1 Overview of the different nitrite-free dry fermented sausages treatments. Nitrite-free dry fermented sausages are based on different meat sources, namely pork (pork), horsemeat (horse) and a 50/50 combination of both meat sources (combi). Different pH conditions are obtained by adding different concentrations of dextrose to the meat batter, 0.00% (high) and 0.70% (low).

meat source treatment	pH treatment	code
Pork	0.00% dextrose	pork-high
	0.70% dextrose	pork-low
horsemeat	0.00% dextrose	horse-high
	0.70% dextrose	horse-low
50/50 combination pork and horsemeat	0.00% dextrose	combi-high
	0.70% dextrose	combi-low

Table 2 pH (*n* = *6*), a_w (*n* = *6*) and weight losses (*n* = *2*) during the production of nitrite-free dry fermented sausages based on pork (pork), horsemeat (horse) and a 50/50 combination of both meat sources (combi). Different pH conditions are obtained by adding different concentrations of dextrose to the meat batter, 0.00% (high) and 0.70% (low).

		production day								
	treatment	**0**	**3**	**21**	**42**	**63**	**84**	**105**	**126**	**168**
pH	**pork-high**	5.36±0.01$_{a,1}$	4.96±0.01$_{e}$	5.22±0.02$_{f}$	5.41±0.01$_{e}$	5.68±0.04$_{d}$	5.60±0.02$_{d}$	5.68±0.06$_{e}$	5.78±0.02$_{e}$	6.03±0.10$_{c}$
(-)	**pork-low**	5.38±0.01$_{a,1}$	4.28±0.02$_{b}$	4.51±0.01$_{d}$	4.59±0.01$_{c}$	4.82±0.05$_{b}$	4.82±0.06$_{b}$	4.89±0.03$_{b}$	5.13±0.02$_{c}$	5.52±0.01$_{b}$
	horse-high	5.40±0.01$_{a,1}$	4.45±0.01$_{c}$	4.43±0.02$_{c}$	4.55±0.02$_{c}$	4.76±0.02$_{b}$	4.88±0.04$_{b}$	5.06±0.03$_{c}$	5.17±0.04$_{c}$	5.47±0.02$_{b}$
	horse-low	5.38±0.02$_{a,1}$	4.12±0.04$_{a}$	4.11±0.01$_{a}$	4.21±0.01$_{a}$	4.28±0.03$_{a}$	4.47±0.03$_{a}$	4.62±0.03$_{a}$	4.72±0.02$_{a}$	5.17±0.07$_{a}$
	combi-high	5.39±0.02$_{a,1}$	4.61±0.01$_{d}$	4.78±0.02$_{e}$	4.99±0.01$_{d}$	5.30±0.08$_{c}$	5.31±0.03$_{c}$	5.45±0.03$_{d}$	5.54±0.03$_{d}$	5.83±0.04$_{c}$
	combi-low	5.34±0.01$_{a,1}$	4.19±0.01$_{a}$	4.26±0.01$_{b}$	4.28±0.01$_{b}$	4.39±0.02$_{a}$	4.76±0.06$_{b}$	4.70±0.02$_{a}$	4.89±0.02$_{b}$	5.12±0.01$_{a}$
weight	**pork-high**	0.00±0.00$_{a,1}$	4.02±0.19$_{a,1}$	15.37±0.87$_{a,1}$	24.87±1.37$_{a,1}$	29.82±1.65$_{a,1}$	33.33±2.17$_{a,1}$	36.27±2.09$_{a,1}$	36.76±2.11$_{a,1}$	40.14±2.11$_{a,1}$
loss	**pork-low**	0.00±0.00$_{a,1}$	3.86±0.52$_{a,1}$	15.22±2.52$_{a,1}$	25.48±0.89$_{a,1}$	31.34±2.01$_{a,1}$	33.20±0.47$_{a,1}$	35.46±0.21$_{a,1}$	35.41±0.11$_{a,1}$	39.05±0.26$_{a,1}$
(%)	**horse-high**	0.00±0.00$_{a,1}$	3.00±0.22$_{a,1}$	15.37±1.18$_{a,1}$	27.58±0.43$_{a,1}$	32.43±0.56$_{a,1}$	35.65±0.36$_{a,1}$	39.02±0.26$_{a,1}$	39.23±0.55$_{a,1}$	43.28±0.84$_{a,1}$
	horse-low	0.00±0.00$_{a,1}$	2.65±0.24$_{a,1}$	13.98±0.37$_{a,1}$	26.26±1.05$_{a,1}$	32.18±0.31$_{a,1}$	36.11±0.43$_{a,1}$	39.05±0.17$_{a,3}$	39.96±0.40$_{a,3}$	43.64±0.10$_{a,3}$
	combi-high	0.00±0.00$_{a,1}$	3.14±0.10$_{a,1}$	15.53±0.51$_{a,1}$	26.97±0.11$_{a,1}$	30.46±0.05$_{a,1}$	33.44±0.49$_{a,1}$	36.97±0.02$_{a,1}$	38.28±0.34$_{a,1}$	41.95±0.54$_{a,1}$
	combi-low	0.00±0.00$_{a,1}$	3.06±0.07$_{a,1}$	14.64±0.04$_{a,1}$	25.75±0.03$_{a,1}$	30.60±0.08$_{a,1}$	34.88±0.40$_{a,1}$	37.37±0.12$_{a,2}$	37.91±0.003$_{a,2}$	41.78±0.03$_{a,2}$
A_w	**pork-high**	0.963±0.001$_{a,1}$	0.960±0.001$_{a}$	0.948±0.001$_{a,1}$	0.933±0.002$_{a,1}$	0.916±0.002$_{b,1}$	0.890±0.002$_{a,1}$	0.882±0.002$_{a,1}$	0.874±0.001$_{bc}$	0.861±0.004$_{a,2}$
(-)	**pork-low**	0.961±0.001$_{a,1}$	0.962±0.001$_{ab}$	0.946±0.001$_{a,1}$	0.928±0.003$_{a,1}$	0.906±0.003$_{a,1}$	0.884±0.004$_{a,1}$	0.876±0.004$_{a,12}$	0.878±0.001$_{c}$	0.851±0.001$_{a,2}$
	horse-high	0.965±0.002$_{a,1}$	0.965±0.001$_{c}$	0.952±0.001$_{a,2}$	0.935±0.001$_{b,1}$	0.914±0.001$_{b,1}$	0.890±0.001$_{b,1}$	0.883±0.001$_{b,1}$	0.874±0.001$_{bc}$	0.845±0.004$_{b,1}$
	horse-low	0.962±0.001$_{a,1}$	0.964±0.0002$_{bc}$	0.950±0.0004$_{a,2}$	0.931±0.001$_{a,1}$	0.902±0.001$_{a,1}$	0.880±0.002$_{a,1}$	0.867±0.001$_{a,1}$	0.859±0.001$_{a}$	0.838±0.005$_{a,1}$
	combi-high	0.965±0.001$_{a,1}$	0.965±0.0002$_{c}$	0.954±0.0003$_{b,2}$	0.936±0.001$_{b,1}$	0.916±0.001$_{b,1}$	0.903±0.002$_{b,2}$	0.888±0.002$_{b,1}$	0.876±0.002$_{c}$	0.850±0.002$_{b12}$
	combi-low	0.964±0.001$_{a,1}$	0.960±0.001$_{a}$	0.950±0.001$_{a,12}$	0.930±0.002$_{a,1}$	0.909±0.001$_{a,1}$	0.890±0.001$_{a,1}$	0.878±0.001$_{a,2}$	0.869±0.001$_{b}$	0.842±0.002$_{a12}$

Data are expressed as means ± SE. If no interaction between meat source and pH condition, same letters indicate no significant differences ($P < 0.05$) between pH treatments within sampling day and same numbers indicate no significant differences ($P < 0.05$) between meat sources within sampling day. If interaction, same letters indicate no significant differences ($P < 0.05$) between all treatments within sampling day.

Table 3 Zn(II)PPIX, PPIX and total heme evolution (*n = 2*) during the production of nitrite-free dry fermented sausages, based on pork (pork), horsemeat (horse) and a 50/50 combination of both meat sources (combi) each at high and low pH conditions (addition of 0.00% and 0.70% dextrose, respectively)

		production day			
	treatment	**0**	**21**	**63**	**168**
Zn(II)PPIX	**pork-high**	6.96±0.01^{a,1}	14.88±1.49^{a,2}	14.23±0.57^{a,2}	11.63±0.91^{a,12}
(nmol/g DM)	**pork-low**	6.55±0.14^{a,1}	8.56±0.08^{a,1}	8.20±0.19^{a,1}	6.78±0.01^{a,1}
	horse-high	8.33±0.24^{a,1}	39.38±4.17^{b,2}	42.31±2.09^{b,2}	59.51±2.02^{b,3}
	horse-low	8.74±0.13^{a,1}	20.92±0.23^{b,2}	23.49±0.98^{c,23}	26.15±2.34^{c,3}
	combi-high	7.43±0.11^{a,1}	38.16±1.13^{b,2}	49.89±0.27^{c,3}	61.20±4.55^{b,4}
	combi-low	7.48±0.03^{a,1}	19.00±0.93^{b,2}	17.29±1.19^{b,2}	16.93±0.33^{b,2}
PPIX	**pork-high**	3.49±0.004^{a,1}	8.57±0.88^{a,2}	12.49±0.86^{a,3}	6.21±2.73^{a,12}
(nmol/g DM)	**pork-low**	3.27±0.07^{a,1}	3.50±0.001^{a,1}	3.21±0.003^{a,1}	3.10±0.06^{a,1}
	horse-high	3.68±0.04^{a,1}	10.29±0.70^{a,2}	10.73±0.48^{a,2}	10.59±0.18^{b,2}
	horse-low	3.68±0.07^{a,1}	4.88±0.04^{a,1}	4.85±0.16^{a,1}	4.91±0.50^{ab,1}
	combi-high	3.41±0.004^{a,1}	9.21±0.69^{a,2}	10.93±2.89^{a,2}	5.75±0.88^{a,1}
	combi-low	3.44±0.01^{a,1}	5.06±0.07^{a,1}	4.59±0.16^{a,1}	5.77±0.74^{b,1}
total heme	**pork-high**	216.54±24.79^{a,2}	165.09±0.57^{a,1}	143.23±8.84^{a,1}	156.43±5.05^{a,1}
(nmol/g DM)	**pork-low**	187.71±6.58^{a,2}	161.15±2.77^{a,12}	153.62±4.53^{a,12}	132.95±5.91^{a,1}
	horse-high	585.54±33.85^{c,4}	497.62±11.93^{c,3}	359.55±14.13^{c,1}	449.48±11.27^{c,2}
	horse-low	565.87±1.62^{c,4}	492.89±13.62^{c,3}	307.81±4.35^{c,1}	437.40±4.16^{c,2}
	combi-high	339.61±38.00^{b,2}	259.91±12.46^{b,1}	246.81±24.75^{b,1}	214.29±7.86^{b,1}
	combi-low	290.60±14.22^{b,1}	296.81±5.19^{b,1}	254.11±5.74^{b,1}	267.03±15.71^{b,1}

Data are expressed as means ± SE. Same letters indicate no significant differences ($P < 0.05$) between meat source within sampling day and pH condition. Same numbers indicate no significant differences ($P < 0.05$) between sampling days within meat source and pH condition. If underlined, significant differences ($P < 0.05$) between pH condition within sampling day and meat source were obtained.

Table 4 Changes in *L, *a** and *b** (*n = 2*) during the production of nitrite-free dry fermented sausages, based on pork (pork), horsemeat (horse) and a 50/50 combination of both meat sources (combi) each at high and a low pH conditions (addition of 0.00% and 0.70% dextrose, respectively)**

		production day			
	treatment	**0**	**21**	**63**	**168**
L*	**pork-high**	59.39±1.60$_{c,2}$	58.31±1.36$_{abc,12}$	58.62±1.73$_{b,12}$	54.80±1.91$_{b,1}$
(-)	**pork-low**	59.67±0.48$_{c,12}$	61.30±0.09$_{c,2}$	59.53±0.20$_{c,12}$	57.50±0.10$_{b,1}$
	horse-high	47.13±0.23$_{a,12}$	50.74±0.74$_{a,2}$	50.32±0.77$_{a,2}$	42.35±1.35$_{a,1}$
	horse-low	45.05±1.49$_{a,1}$	51.93±0.06$_{a,2}$	48.06±0.08$_{a,1}$	46.92±0.95$_{a,1}$
	combi-high	53.33±0.60$_{b,1}$	55.66±0.40$_{b,1}$	55.45±0.35$_{b,1}$	50.30±0.20$_{b,1}$
	combi-low	52.87±1.07$_{b,2}$	55.99±0.94$_{b,2}$	55.48±0.41$_{b,2}$	49.02±1.28$_{a,1}$
a*	**pork-high**	15.36±0.52$_{a,3}$	10.10±0.24$_{b,1}$	10.70±0.75$_{c,1}$	12.74±0.90$_{b,2}$
(-)	**pork-low**	15.52±0.51$_{a,3}$	9.58±0.27$_{c,1}$	9.28±0.58$_{c,1}$	11.64±0.27$_{c,2}$
	horse-high	18.64±0.05$_{a,3}$	6.18±0.34$_{a,2}$	3.21±0.02$_{a,1}$	3.70±0.48$_{a,1}$
	horse-low	20.15±0.45$_{b,3}$	6.09±0.43$_{a,2}$	2.82±0.16$_{a,1}$	3.51±0.25$_{a,1}$
	combi-high	17.10±0.16$_{a,3}$	9.55±0.32$_{b,2}$	7.46±0.13$_{b,1}$	9.91±0.36$_{b,2}$
	combi-low	16.27±0.26$_{a,3}$	8.39±0.003$_{b,2}$	4.96±0.32$_{b,1}$	5.99±0.28$_{b,1}$
b*	**pork-high**	20.77±0.16$_{c,4}$	10.73±0.07$_{c,1}$	12.33±0.42$_{c,2}$	13.16±0.45$_{c,3}$
(-)	**pork-low**	20.84±0.35$_{b,4}$	8.45±0.06$_{c,1}$	10.78±0.42$_{c,2}$	11.19±0.22$_{c,3}$
	horse-high	20.12±0.51$_{a,3}$	6.95±0.45$_{a,1}$	8.72±0.03$_{a,2}$	7.32±0.06$_{a,1}$
	horse-low	20.63±0.22$_{a,3}$	6.20±0.31$_{a,1}$	7.53±0.39$_{a,2}$	8.11±0.41$_{a,2}$
	combi-high	20.65±0.40$_{b,3}$	7.82±0.26$_{b,1}$	10.51±0.30$_{b,2}$	9.48±0.07$_{b,2}$
	combi-low	19.68±0.16$_{a,4}$	6.99±0.26$_{b,1}$	8.97±0.29$_{b,3}$	8.66±0.17$_{b,2}$

Data are expressed as means ± SE. Same letters indicate no significant differences ($P < 0.05$) between meat source within sampling day and pH condition. Same numbers indicate no significant differences ($P < 0.05$) between sampling days within meat source and pH condition. If underlined, significant differences ($P < 0.05$) between pH condition within sampling day and meat source were obtained.

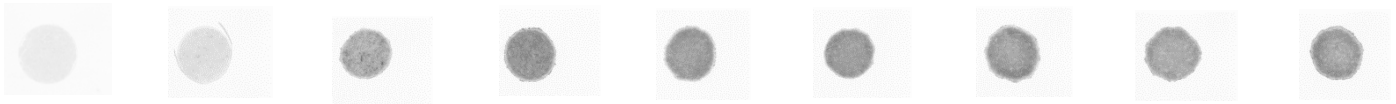
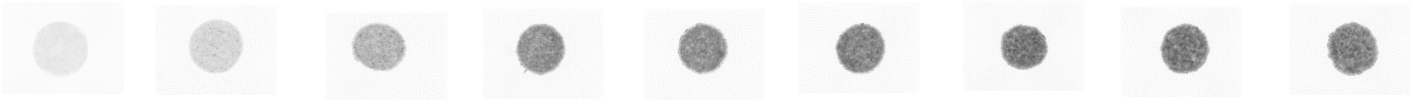
meat source	pH treatment	production day								
		0	3	21	42	63	84	105	26	168
pork	high									
	low									
horse	high									
	low									
combi	high									
	low									

Figure 1 Evolution of Zn(II)PPIX and/ or PPIX during the production of nitrite-free dry fermented sausages based on pork (pork), horsemeat (horse) and a 50/50 combination of both meat sources (combi) using a fast screening method. Different pH conditions are obtained by adding different concentrations of dextrose to the meat batter, 0.00% (high) and 0.70% (low).